

# Isolation of Natural Arachidonic Acid as Its Methyl Ester<sup>1</sup>

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IN a recent publication (1) this laboratory reported the isolation of pure natural linoleic and linolenic acids as their methyl esters by adsorption fractionation on silicic acid. Tobacco seed oil and linseed oil, respectively, were the sources of these acids. The natural acid esters differed significantly from corresponding debromination acid esters in the intensity of ultra-violet absorption at their maxima under the conditions of the alkali-isomerization spectrophotometric method of analysis. In view of these findings, revised constants were suggested for this method when it is used for analyzing natural oils. Since many animal fats, particularly glandular fats, contain significant amounts of arachidonic acid in addition to linoleic and linolenic acids, it is important to extend this investigation to the isolation of natural arachidonic acid and study of the ultra-violet absorption characteristics of its alkali-isomerized product.

The presence of eicosatetraenoic acid in liver lipids was first shown in 1909 by Hartley (2) by identification of bromination and oxidation products. Lewkowitsch (3) suggested the name arachidonic acid for this compound. Later work has established the presence of this acid in phospholipids of liver, brain, and egg yolk. In fact, it has since been shown that small amounts are present in almost every animal tissue and glandular fat. A review of the literature is given by Ralston (4).

Brown and associates (5, 6) prepared arachidonic acid by bromination-debromination techniques and also reported its preparation in 90-95% purity by fractional crystallization and distillation (7, 8). Recently White and Brown (9) announced the preparation of methyl arachidonate from beef suprarenals by adsorption fractionation on alumina. They also reported evidence for the presence of a  $C_{20}$  acid having five double bonds. Their complete description of this work however has not been published. No information was given in the report regarding the ultra-violet absorption characteristics of their preparations after isomerization with alkali. Such information would appear essential for establishing homogeneity of the acids.

In the present paper the authors describe the preparation of pure methyl arachidonate in which the principal fractionation was accomplished by chromatographic absorption treatment on silicic acid columns. Some initial concentration of arachidonic acid was achieved by low-temperature crystallization, and the final purification was accomplished by fractional distillation in vacuo.

## Experimental and Results

Since the work of Brown and co-workers indicated that beef suprarenals or adrenal glands are a satis-

factory source of arachidonic acid, these glands were used as a starting material in the present work.

*Preliminary Adsorption Fractionation Experiments.* Our preliminary experiments on fractionation of methyl esters of beef suprarenals on silicic acid columns showed that these lipids are a complex mixture containing dienoic, trienoic, tetraenoic, and pentaenoic acid esters. This is in agreement with observations of White and Brown (9). It was found necessary to follow the fractionation by spectrophotometric examination because in several instances fractions were obtained which had iodine values in agreement with the theoretical iodine value for methyl arachidonate but which spectrophotometrically showed considerable amounts of pentaenoic acid esters, as indicated by the appearance of an absorption maximum at 346  $m\mu$  after alkali isomerization.

During this preliminary work small fractions having iodine values of 310-312 (theory for methyl arachidonate, 318.8) were obtained free of pentaene. Rechromatographing these fractions however failed to effect further purification. It appeared therefore that to purify further means other than adsorption fractionation on silicic acid should be tried. Fractional distillation under high vacuum gave further fractionation.

In the adsorption fractionation on silicic acid columns, after most of the methyl arachidonate was eluted, fractions high in pentaene were removed. A small fraction, iodine value 390.9, was obtained which had the following specific extinction coefficients<sup>3</sup>: 233  $m\mu$ , 54.4; 268  $m\mu$ , 36.9; 315  $m\mu$ , 21.4; 346  $m\mu$ , 11.1. The coefficients were determined after isomerization in 11% KOH-glycerol under nitrogen for 45 minutes at 180° C. The theoretical iodine value for the methyl ester of eicosapentaenoic acid is 401.

The samples of methyl arachidonate of about 90% purity obtained in these preliminary experiments were too small to warrant further attempts at purification. Hence the fractionation was repeated with larger quantities of starting material. This fractionation will be reported in greater detail.

*Extraction of Lipids From Adrenal Glands.* Fresh beef adrenal glands, approximately 30 pounds, were obtained at a local abattoir. After removal of adhering body fat, the glands were minced in a food grinder, extracted several times with ethanol at room temperature, and then thoroughly extracted with successive portions of peroxide-free ethyl ether. The alcohol extracts were concentrated, and the fatty aqueous residue was extracted with ethyl ether. All ether extracts were then combined, and the solvent was removed. The total lipid residue weighed 558 grams; the iodine value (I.V.) was 83.4. Arachidonic acid content estimated from spectrophotometric examination was 8-9%.

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<sup>3</sup> In this paper, specific extinction coefficient  $k = D/bc$ , where  $D$  = spectral density of the solution (compared with solvent),  $b$  = length of cell, and  $c$  = concentration in grams/liter.

TABLE I  
Adsorption Fractionation on Silicic Acid of a Concentrate of  
Methyl Esters of Polyunsaturated Acids

Fraction Number	Volume of Eluate <sup>a</sup>  ml.	Fraction	
		Weight  g.	Iodine Value  (Wt%)
Original.....	.....	50.04	161.1
1.....	8000	0.77	47.4
2.....	2400	1.71	41.9
3.....	2300	3.48	57.0
4.....	2400	4.67	74.0
5.....	2400	5.02	78.8
6.....	2700	4.53	83.2
7.....	3200	3.42	84.9
8.....	2000	2.28	85.4
9.....	2200	2.32	97.9
10.....	2500	2.80	140.7
11.....	2500	2.04	158.4
12.....	2000	0.88	166.9
13.....	2400	0.50	174.0
14.....	2500	0.35	178.2
15.....	3100	5.06	282.1
16.....	2700	6.50	307.0
17.....	3000	2.63	353.3
18.....	2300	0.97	358.1

<sup>a</sup> Petroleum ether containing 0.25, 0.5, and 1.0% ethyl ether was used to elute fractions 1 to 10, 10 to 13, and 13 to 18, respectively.

**Concentration of Arachidonic Acid.** The total lipids were saponified with alcoholic-KOH under nitrogen for 3.5 hours at reflux temperature. The fatty acids recovered weighed 460 grams. (A white precipitate weighing 63 grams which was insoluble in petroleum ether and in water was also obtained.) The fatty acids, 460 grams, were crystallized from 7 liters of acetone at 0° C. and filtered. The filtrate was then held at -18° C. and filtered. After this second filtrate was concentrated to about 3.5 liters, it was held at -40° C. and filtered. The three precipitates, weighed 236 grams and consisted principally of saturated and oleic acids.

The filtrate residue, 217 grams, was made alkaline with alcoholic-KOH solution and extracted with ether to remove unsaponifiable matter. The material thus removed, 25 grams, I.V., 145.7, contained 42.7% cholesterol. The recovered acids, 192 grams, were treated with anhydrous methanol and dry HCl to form their methyl esters. The yield of methyl esters was 194 grams, I.V., 161.1. After isomerization in glycerol-KOH under nitrogen for 45 minutes at 180° C.,  $k_{233} = 25.0$ ;  $k_{268} = 14.3$ ;  $k_{315} = 5.5$ ;  $k_{346} = 0.96$ . The content of methyl arachidonate was estimated to be about 25%. This preparation was used in the fractionation on silicic acid.

**Adsorption Fractionation on Silicic Acid.** The same type of column and treatment of the column as described in a previous publication (1) was used in this work. The glass column was 6.3 cm. in diameter and 104 cm. in height. It was packed with 800 grams of silicic acid-filter aid mixture (80 parts silicic acid, 20 parts filter aid) to a depth of 73.5 cm. The column adsorbent mixture was heated to 70-75°C. for 3.5 hours, with slow passage of pure nitrogen, then allowed to cool to room temperature in the stream of gas. After the mixture was wet with 1500 ml. of petroleum ether (boiling range 30-50° C.) containing 0.25% ethyl ether, 50 grams of the methyl esters, I.V., 161.1, dissolved in 250 ml. of the same solvent were added. The sample was protected from light during the experiment by wrapping several thicknesses of dark-colored paper around the glass column. The receiver was shielded from light by wrapping it with black cloth. Successive elutions were made with petroleum ether containing 0.25, 0.5, and 1.0% ethyl

ether (Table I). By adjusting the rate of percolation and using a receiver and separatory funnel of adequate size, the fractionation was continued during the night without attention. Other data pertaining to the fractionation are also given in Table I.

In addition to iodine values, spectrophotometric examinations were made on certain samples. This was particularly important in the fractions whose iodine numbers approached the theoretical value for methyl arachidonate, 318.8, for example, fractions 15 and 16, I.V., 282.1-307.0, to determine whether they contained any pentaene. Fractions 17 and 18, whose iodine values were higher than that of methyl arachidonate, contained considerable amounts of a pentaene acid, as indicated by spectrophotometric examination after alkali-isomerization. Table II shows these data.

TABLE II  
Specific Extinction Coefficients of Certain Fractions  
Shown in Table I

Fraction Number (Table I)	Iodine Value  (Wt%)	Specific Extinction Coefficients <sup>a</sup>			
		$k_{233}$	$k_{268}$	$k_{315}$	$k_{346}$
Original.....	161.1	25.0	14.3	5.5	0.96
8.....	85.4	2.1	0.0	0.0	0.0
11.....	158.4	49.9	6.9	1.2	0.0
15.....	282.1	58.2	36.4	12.0	0.0
16.....	307.0	53.7	45.8	17.4	0.0
17-18.....	355.1	49.8	35.0	21.8	8.7

<sup>a</sup> Isomerized in 11% KOH-glycerol under nitrogen for 45 minutes at 180° C.

It is apparent that up to fraction 8, saturated esters and esters of monoethenoid acids were being removed. Fractions 8-11 inclusive contained increasing amounts of dienoic and trienoic acid esters. Fractions 12-16 inclusive contained increasing amounts of tetraenoic acid esters but no pentaene. Fractions 17 and 18 contained large amounts of pentaene.

From two similar adsorption fractionations, the following fractions rich in methyl arachidonate were obtained: 5.7 grams, I.V., 309.4; 3.2 grams, I.V., 308.9; and 1.2 grams, I.V., 303.7. All these fractions showed absence of pentaene by spectrophotometric examination and were combined with fraction 16 (Table I) for further adsorption fractionation. The new column was 3.8 cm. in diameter and 81 cm. in height and was filled with 250 grams of silicic acid-filter aid mixture to a depth of 58 cm. The treatment of the mixture was essentially the same as that previously described. The results in Table III show that

TABLE III  
Adsorption Fractionation on Silicic Acid of Methyl  
Arachidonate-Rich Fractions

Fraction Number	Volume of Eluate <sup>a</sup>  ml.	Fraction	
		Weight  g.	Iodine Value  (Wt%)
Original.....	.....	16.00	303-309
1.....	1100	0.18	13.0
2.....	1600	0.34	291.9
3.....	3200	2.79	305.1
4.....	1800	4.47	309.6
5.....	1700	3.43	309.8
6.....	1700	1.63	311.9
7.....	1700	1.00	305.7
8.....	1900	0.49	310.5
9.....	6100	0.48	307.1
10.....	1700	0.65	311.2

<sup>a</sup> Petroleum ether containing 0.25% ethyl ether was used to elute all the fractions except fraction 10, which was removed with 20% ethyl ether.

although some further fractionation was achieved, complete separation of methyl arachidonate from the impurities was not accomplished.

**Fractional Distillation.** Fractions 4 and 5, Table III, were fractionally distilled through an electrically heated Vigreux column, 3.1 cm. in diameter and 21 cm. in height, at 0.015 mm. pressure. Table IV shows the fractions obtained and their analyses.

TABLE IV  
Fractional Distillation of Fractions 4 and 5, Table III

Fraction Number	Weight	Iodine Value	Specific Extinction Coefficients <sup>a, b</sup>		
			k <sub>233</sub>	k <sub>258</sub>	k <sub>315</sub>
Fractions 4 and 5, Table III	7.24	Wtgs 309.6- 309.8	.....	.....	.....
1.....	1.58	305.4	51.7	43.3	16.8
2.....	1.17	310.4	52.7	45.0	17.8
3.....	2.13	313.5	52.3	45.0	19.1
4.....	1.51	316.1	53.0	45.6	19.7
5.....	0.60	315.7	52.1	45.1	19.7
Residue.....	0.60	308.0	.....	.....	.....

<sup>a</sup> Isomerized in 11% KOH-glycerol under nitrogen for 45 minutes at 180° C.

<sup>b</sup> In all fractions, k<sub>315</sub> = 0.0.

The results show that the impurity was being removed in fractions 1, 2, and 3. The iodine values of fractions 4 and 5 are within experimental error of the theoretical iodine value for methyl arachidonate. The specific extinction coefficients show a leveling off at 315 mμ, fractions 4 and 5 having identical values. Other analytical values obtained on fraction 4 were: saponification equivalent 316.3, theory 318.5; neutralization equivalent of acid 303.8, theory 304.4; N<sub>D</sub><sup>20</sup> = 1.47986. A hydrogenated portion melted at 45.0°C. (values in literature for methyl arachidate, 44.4-44.7°, 45.0°, 46.6° C.) and gave the same x-ray diffraction pattern as a known specimen of pure methyl arachidate.

In a distillation of the remaining fractions shown in Table III whose iodine values ranged from 305 to 311 a fraction, I.V., 314.9, was obtained. A portion of the distilled material, I.V., 314.9, on further adsorption fractionation yielded a small fraction, I.V., 318.0. Both fractions on alkali isomerization had essentially the same specific extinction coefficients as fractions 4 and 5, Table IV. All purified specimens of methyl arachidonate, I.V., 314.9-318.0, were reserved for a more detailed study of their ultra-violet absorption characteristics under various conditions of alkali-isomerization. The results of this study will be reported in another paper (10).

Attempts were made to determine the identity of the impurity removed by distillation. Fraction 1, Table IV, had a saponification equivalent of 309.1. After hydrogenation of the ester and crystallization of the saturated product, the first crop of crystals was identified by x-ray diffraction as methyl arachidate; the second crop was a mixture of methyl stearate and methyl arachidate. From considerations of the distillation data and extinction coefficients in Table IV, it seems probable that the C<sub>18</sub> acid ester impurity was

chiefly methyl linolenate. These data however do not preclude the possible presence of methyl eicosatrienoate. Calculations of the composition of the fractions shown in Table IV, in which the extinction coefficients of the natural linoleic, linolenic, and arachidonic acids (10) are used, indicate that fractions 1, 2, and 3 contained 9.7, 8.3, and 1.8%, respectively, of methyl linolenate.

The fractions that contained pentaene esters were reserved for further work.

## Summary

Fresh beef suprarenal glands were ground and extracted thoroughly with alcohol and then with ethyl ether. After removal of solvent the total lipid residue was saponified, and the fatty acids were recovered by extraction. The less unsaturated acids were removed by crystallization from acetone at -40°C. At this stage the filtrate contained essentially all the arachidonic acid originally present in the glands and also unsaponifiable matter. After the unsaponifiable material was removed, the arachidonic acid content of the concentrate was about 25%. These unsaturated acids were converted to their methyl esters and fractionated on a silicic acid adsorption column. The progress of the adsorption fractionation was followed by spectrophotometric examination and determination of iodine values of the eluted fractions. Methyl arachidonate of 90% purity was obtained by this means. It was further purified by fractional distillation in vacuo. The final product had an iodine value of 316.1; theory, 318.8. The purity of this preparation was further established by spectrophotometric examination, by saponification equivalents, mean molecular weight, and by x-ray diffraction patterns and melting points of a completely hydrogenated portion.

Evidence of acids of greater unsaturation than arachidonic acid in suprarenal lipids was also clearly established by spectrophotometric examination. A fraction was obtained which was estimated to contain about 80-85% of a C<sub>20</sub> acid with five double bonds.

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